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Microbiological and molecular analysis of endodontic infections

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This article reviews microbiological and molecular methods used to study the microorganisms associated with endodontic infections. Over 500 species of bacteria have been cultivated from the oral cavity. Endodontic infections are polymicrobial with usually from 3–12 species cultivable from either infected root canals or periradicular abscesses. Molecular methods are able to detect and identify many additional species of bacteria associated with endodontic infections. Molecular methods provide precise identification of the microbes at the DNA level and detection of microbes that are not cultivable. Future research will detect and identify other as yet unknown species of bacteria, viruses, and fungi involved in endodontic infections. Which microorganisms produce virulence factors associated with serious endodontic infections will also be better defined using molecular methods. An increase in knowledge and understanding of the organisms associated with endodontic infections will improve our ability to clinically manage endodontic infections.

The oral cavity is similar to other sites of the body in that it is colonized with 'normal flora' in a symbiotic relationship. Indeed, it has been estimated that the average human body is colonized by 10 times more bacteria (10^{14} bacteria) than there are mammalian cells (10^{13} cells) (1). The microbial population and the surrounding environment are together known as the ecosystem. The establishment of microorganisms in a host is termed colonization. Permanent colonization in a symbiotic relationship results in the establishment of normal flora. Microbial colonization of the newborn is a stimulus for the development of the immune system. If the normal flora is provided the right conditions and gain access to a normally sterile tissue such as the dental pulp or periradicular tissues, they become opportunistic pathogens. The degree of pathogenicity is termed virulence. The host's response to a microbial infection may be both non-specific inflammation and/or specific immunological reactions.

An infection is produced if the invasion of microbes produces damage to tissue. Endodontic disease (pulpal and periradicular) is the result of both the pathogenic effects of the microbes and the response of the host. The goal of clinicians is to disrupt and destroy the microbial ecosystem associated with the disease process. This article highlights the possibilities and limitations of clinical sampling, culturing and identification (characterization) of microbes. Great progress has been made in detecting and identifying the microbes involved in endodontic infections.

Microorganisms were observed in samples from teeth by Leeuwenhoek (2) soon after he invented the microscope in 1684. Since Babylonian times, it was believed that a 'tooth worm' lived in the hollow portion of the tooth and caused decay. Leeuwenhoek challenged the 'tooth worm' theory of decay by identifying worm-infested cheese that he thought may be the source of disease (3). Leeuwenhoek also described microorganisms that he scraped from teeth as 'cavorting beasties' (2). Since 1890, when Miller (4) first observed microorganisms associated with pulp tissue, microorganisms have been implicated in infections of endodontic origin. In 1965 Kakehashi et al. (5) demonstrated that pulpal necrosis and the development of periapical inflammatory lesions only occur after exposure of the dental pulp to the oral cavity in conventional rats with normal oral microorganisms and not in germ-free rats. Microbiological studies of endodontic infections through the 1960s used primarily microscopy, aerobic culturing techniques, and biochemical reactions to detect and identify microbes (6–8). In the 1970s, it was then determined that most bacteria in endodontic infections are strict anaerobes (9–12). The diversity of the polymicrobial endodontic infections has been well established with from three to 12 species of microbes isolated from the majority of endodontic infections (13–19).

Normal oral flora

Oral microflora are very diverse with over 500 species cultivable and many others not cultivable. The microbes of the oral cavity consist of bacteria, fungi, viruses, and occasionally protozoa. The diversity is related to varied habitats in the oral cavity with a variety of nutrients. Dental plaque associated with caries and periodontal disease has gradients of oxygen tension and pH that provide conditions for the growth and survival of a wide spectrum of bacteria. The large population of normal oral flora makes it difficult for invading pathogens to compete for nutrients and receptor sites. In addition, some bacteria produce antimicrobial substances to protect their habitat from invasion by other organisms. By-products produced by one species of bacteria may be a nutrient for some species of bacteria while being toxic for other species. Bacteriocins are antibiotic-like proteins produced by bacteria to inhibit other species of bacteria.

The normal flora covering all the surfaces of our bodies accounts for more clinical disease than disease by recognized pathogens. This occurs when the microorganisms are displaced into an abnormal site. For example, a bacteremia may lead to endocarditis in a susceptible individual. Antibiotic-associated colitis is produced when normal bowel microflora are diminished allowing the overgrowth of the antibiotic resistant *Clostridium difficile*. When patients are traumatized or medically compromised, normal flora can overgrow and become pathogenic.

Dental caries remains the most common cause of endodontic disease. In addition to caries, pathways for the entry of microorganisms into the pulp space include direct pulp exposure (e.g. caries, trauma, dental procedures), dentinal tubules, lateral/accessary/furcation canals, and anachoresis. Once the pulp is necrotic and the odontoblastic processes undergo autolysis, patent dentinal tubules (dead tracts) are traversed by microorganisms and infect the root canal system (20). When the pulpal tissue becomes necrotic, it loses its blood supply and the root canal system becomes a reservoir for microorganisms and their by-products. Because of the lack of circulation within the necrotic pulp, the root canal system becomes a sanctuary for microorganisms from the immune system.

Table 1. Characteristics often used for microbial classification

Characteristic	Examples
Cellular morphology	Shape, Gram stain, flagella, spores, size
Colonial appearance	Pigment, hemolysis, shape
Carbohydrate fermentation	Acid or gas production
Amino acid hydrolysis	Ammonia production
Fermentation products	Butyrate, lactate, acetate
Preformed enzymes	Glycosidases
Antigens	Monoclonal/polyclonal antibodies
Lipids	Menaquinones, long chain fatty acid
Enzyme profile	Malate dehydrogenase
Peptidoglycan	Amino acid composition
DNA	Base composition, base sequence homology

Bacterial taxonomy

Because of the diversity of microorganisms, a taxonomic system for classification has been devised based on their characteristics. Generally strains within a species share more than 70% of the genetic sequence. In addition to genetic comparisons, other methods referred to as chemotaxonomy include: analysis of membrane lipids, peptidoglycan structure, enzyme mobilities, and whole-cell protein patterns derived from polyacrylamide gel electrophoresis. Once microbes are classified, identification methods are devised. Table 1 shows the most commonly used characteristics for microbial classification and identification.

Based on recent innovations, the taxonomy of many oral microorganisms including bacteria associated with endodontic infections has been changed. Although these are improvements in classification, the changes have produced difficulties in interpreting older publications that used outdated nomenclature. For example, *Streptococcus sanguis* has recently had its description narrowed to make it distinct from other species (e.g. *S. gordonii*). As a result, previous investigations are difficult to relate to more recent studies. It is now recommended that the group of streptococci previously referred to as 'viridans streptococci' should not be used because it includes several species of streptococci with different properties. In addition, taxonomic changes have removed species of *Enterococcus* and *Lactococcus* from the genus *Streptococcus* (21–24). Great care must be taken when reading outdated endodontic literature with regard to the names of bacteria that remain of interest today. Many old species have been reclassified and novel species have been proposed. Taxonomic changes are based on genetic relatedness, which does not necessarily correspond with such phenotypic characteristics such as Gram-staining, nutrient/atmospheric requirements, and cell morphology. Phylogenetic studies have revealed that all living organisms fall into three primary domains – Bacteria, Eucarya, and Archaea. Bacteria are in the following six phyla: *Actinobacteria*, *Fusobacteria*, *Proteobacteria*, *Bacteroides*, *Firmicutes*, and *Spirochaetes* with the three later phyla containing the majority of the bacterial identified in endodontic infections (25).

Endodontic infections are polymicrobial infections consisting primarily of obligate anaerobes and facultative anaerobes (10, 14, 26–28). Gram-negative 'black-pigmented bacteria' have received a lot of attention in the field of endodontics. Depending on growth media and conditions, black-pigmented bacteria may actually range in color from tan to black. In addition, some strains of black-pigmented bacteria may undergo spontaneous mutation and lose black pigmentation in the laboratory (Fig. 1). The loss of black pigmentation in Fig. 1 was associated with the capability of hemolysis of the blood agar. Hemolysis is likely a virulence factor. Because the so-called black-pigmented bacteria vary from tan to black depending on the strain, media, and culture conditions, it has been recommended to refer to these organisms as dark-pigmented bacteria (29). The anaerobic genus *Bacteroides* has undergone major taxonomic revision that changed the nomenclature for the dark-pigmented bacteria associated with infections of endodontic origin. These species are all non-motile, non-sporeforming, Gram-negative rods. Because all of the dark-pigmented species were classified as a subspecies of *Bacteroides melaninogenicus* prior to 1977 and because of further characterization of the species, a significant problem in interpreting the literature exists. In 1984, a new species called *B. endodontalis* was proposed based on two asaccharolytic dark-pigmented strains isolated by Sundqvist (10) and a third asaccharolytic strain that was isolated by Van Steenbergen et al. (30–32). Van Steenbergen et al. (33) demonstrated that *B. endodontalis* has negligible DNA homology with either *B. asaccharolyticus* or *B. gingivalis*. Nomenclature changes for dark-pigmented bacteria as occurred from 1977 to 1988 are shown in Table 2.

In 1988, Shah and Collins (34) proposed the reclassification of *B. asaccharolyticus*, *B. gingivalis*, and *B. endodontalis* into a new genus called *Porphyromonas*. Reclassification was based on the fact that these asaccharolytic dark-pigmented bacteria form a homogeneous group that differs remarkably in biochemical

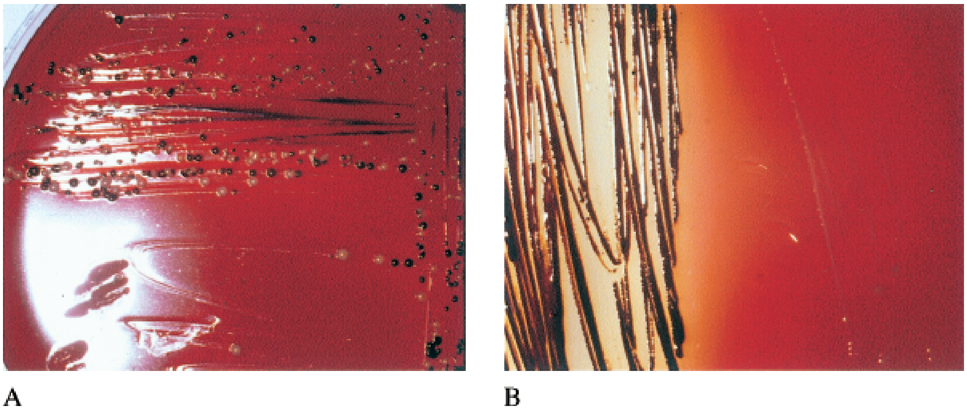

Fig. 1. (A) The subculture of a black-pigmented colony produced many colonies that are tan in color without the black pigmentation, consistent with a mutation of cells in the colony being cultured. (B) When a black colony was subcultured it bred true with hemolysis of the blood in the agar while a tan colony bred true but without hemolysis.

Table 2. Changes in nomenclature for dark-pigmented bacteria (1977–1984)

Bacteroides melaninogenicus subspecies *asaccharolyticus*
B. asaccharolyticus
B. gingivalis (1981)
B. endodontalis (1984)
B. melaninogenicus subspecies *melaninogenicus*
B. melaninogenicus
B. denticola
B. loescheii
B. melaninogenicus subspecies *intermedius*

Table 3. Nomenclature for human species of dark-pigmented bacteria

Dark-pigmented *Porphyromonas* (asaccharolytic *Bacteroides* species)
Porphyromonas asaccharolyticus (usually non-oral)
Porphyromonas gingivalis
Porphyromonas endodontalis
Dark-pigmented *Prevotella* (saccharolytic *Bacteroides* species)
Prevotella melaninogenica
Prevotella denticola
Prevotella loescheii
Prevotella intermedia
Prevotella nigrescens

and chemical properties from the type species of *Bacteroides* (*B. fragilis*). In addition, the saccharolytic dark-pigmented *Bacteroides* were reclassified for similar reasons in 1990 into a new genus called *Prevotella* (35). In 1994, *Prevotella nigrescens* was separated from the species *Prevotella intermedia* (36). Ten species of dark-pigmented bacteria have now been isolated from humans and several other species have been isolated from animals (Table 3).

In addition to dark-pigmented *Prevotella*, non-pigmented *Prevotella* have been isolated from infections of endodontic origin. They include: *Prevotella buccae*, *Prevotella bivia*, *Prevotella oralis*, *Prevotella oris*, *Prevotella oulorum*, and *Prevotella ruminicola*. Other non-pigmented bacteria in the genus *Bacteroides* have had taxonomical changes. For example, *B. forsythus* is now known as *Tannerella forsynthensis* and *B. gracilis* is now *Campyobacter gracilis. B. pneumosintes* was changed to *Dialister pneumosintes* but further phylogenetic analysis showed that it belonged to the *Firmicutes* phylum. Thus considering all the changes in the taxonomy of the dark/light-pigmented '*Bacteroides/Porphyromonas/Prevotella*' it is difficult to evaluate old microbial publications. This is true for numerous other microorganisms associated with endodontic disease (25).

Sensitivity and specificity

Diagnostic microbial research continues to receive a lot of attention in endodontics. Identification of the organisms causing the infectious process remains a goal both to understand the disease process and to provide effective antimicrobial and supportive treatment. Because endodontic infections are polymicrobial, the detection and identification of all associated organisms have been difficult and produced a great deal of confusing data. No absolute correlation has been made between any specific microbial species or combination of species with clinical signs and symptoms (14, 16–19, 37–55).

Sensitivity is a parameter that can be used to determine the relative effectiveness of a diagnostic test. In the field of microbiology, analytical sensitivity is the ability of a test to detect small quantities of an organism or substance in a sample. Specificity is the ability to identify the organisms correctly. For the detection of microbes, microscopy and culturing have low sensitivity and specificity when compared with immunoassays and molecular methods. Even in state-of-the-art microbiology laboratories, many species remain uncultivable. However, culturing is considered the 'gold standard' for microbial research because if an organism can be cultivated, it is available for research studies (e.g. antibiotic susceptibility) and can be tested with Koch's postulates to determine if the organism causes a specific disease.

Koch's postulates

1. The microorganism occurs in every case of the disease in question and under circumstances, which can account for the pathological changes and clinical course of the disease.
2. The microorganism occurs in no other disease as a fortuitous and non-pathogenic parasite.
3. After being fully isolated from the body and repeatedly grown in pure culture, the microorganism can induce the disease anew.

Immunoassays and molecular methods have greater sensitivity and specificity than culturing in the ability to detect an organism; however, the organism is not available for further research.

The terms sensitivity and specificity are also epidemiological terms. Ideally, a test for a pathogen or combination of pathogenic microbes would always be positive in the presence of specific signs or symptoms (true positive) and be absent when the signs and symptoms are not present (true negative). Epidemiological 'sensitivity' is the probability that a test will be positive in the presence of a pathogen:

$$\text{Sensitivity} = \frac{\text{True positive}}{\text{True positive} + \text{Flase negative}} \times 100\%.$$

Epidemiological specificity is the probability that a test will be negative if the pathogen in not present:

$$\text{Specificity} = \frac{\text{True negative}}{\text{True negative} + \text{False postive}} \times 100\%.$$

Clinical sampling, microscopic examination, and culturing of bacteria

When clinical collection of a microbial sample is undertaken, it is important to avoid microbial contamination. An aseptic sample from an infected root canal system is obtained by isolating the tooth with a rubber dam and disinfecting the tooth and surrounding dam (56). The access opening is accomplished with sterile burs. Sterile paper points or sterile instruments are used to remove the sample from the root canal and the sample is placed in pre-reduced transport fluid for delivery to the laboratory (57, 58). Uncontaminated microbial samples from facial swellings of endodontic origin are obtained by needle aspiration following surface disinfection of the mucosa (59). Following aspiration, any air in the syringe should be removed and the sample injected into an anaerobic transport vial for delivery to the laboratory.

Microscopy gives rapid and inexpensive information but morphologic characteristics are usually not adequate to identify an organism at the species level. The Gram stain has been important for initial morphological characterization of the bacteria in an infection and therapeutically because Gram-positive and Gram-negative bacteria may have different susceptibilities to antibiotics. The Gram stains have been used to determine initial antimicrobial therapy until more definitive tests are completed. A Gram stain does require over 1×10^4 bacteria/mL. However, at best a Gram stain only reveals the shape of the microbes and

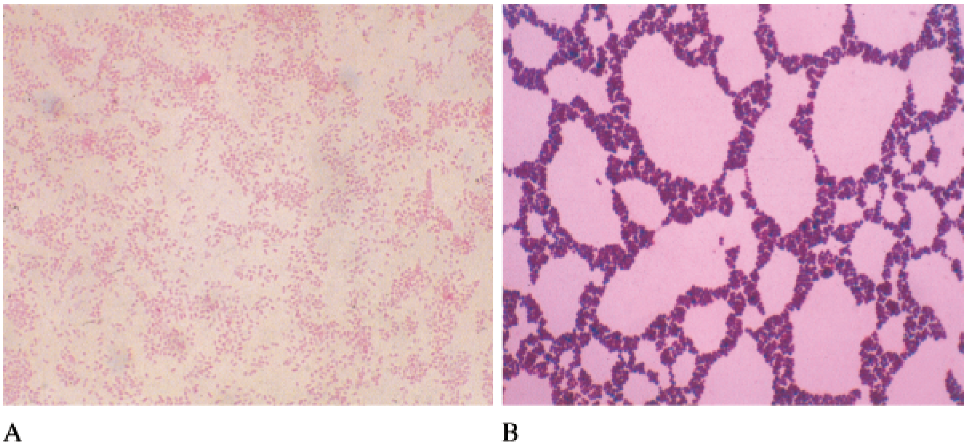

Fig. 2. (A) A Gram-negative strain of *Porphyromonas endodontalis.* (B) A Gram-positive strain of *Peptostreptococcus anaerobius.*

the Gram reaction (Fig. 2). Several Gram-positive species of bacteria are often Gram variable. Gram-negative bacteria are very difficult to see against a background of organic matter. Although a Gram stain gives limited information, it may demonstrate organisms that do not grow in the culture media employed. Phase contrast or dark-field microscopy may be used to examine a clinical sample suspended in water on a glass slide and covered with a thin coverslip. The specimens are examined using a magnification of ×400 or ×1000. Phase contrast or dark-field microscopy are especially helpful in determining motility of organisms and the presence of spirochetes (60). However, microscopy does not give information about the individual species or clonal types within the species.

Labile anaerobic organisms may not survive transport to the laboratory and some microbes may be uncultivable in the media used in the lab. Anaerobic transport vials must be used to transport the clinical sample to the laboratory while the organisms are still cultivable (57, 61). Organisms that are either not viable or uncultivable may be detected and identified using immunoassays and molecular methods. Definitive identification of the infective organisms using conventional methods requires growth of the organism in pure culture. Microbes grown on solid culture media provide information that includes colony size, shape, color, hemolytic reactions, odor, and other metabolic properties. For polymicrobial infections usually both enriched non-selective media and selective media are employed. For example, Columbia rabbit blood agar supplemented with hemin and menadione is an enriched non-selective media used to cultivate dark-pigmented bacteria plus many other species found in endodontic infections. An example of a selective media for Fusobacteria is CVE media that contains crystal violet and erythrocin that inhibits most other species of oral bacteria (61). The culture environment is also important. Aerobes require oxygen while facultative anaerobes are able to grow in the presence or absence of oxygen. In contrast, strict anaerobes such as *Porphyromonas endodontalis* are inhibited or killed by the presence of oxygen (14). Incubation of anaerobes is usually at 36–37°C in an anaerobic chamber or anaerobic jar with an atmosphere of 85% N_2, 5% CO_2, and 10% H_2. Bacteria in a polymicrobial endodontic infection must be subcultured to get the organisms in pure culture for identification.

Identification of bacteria using metabolic properties

Numerous methods have been used for the identification of bacteria. The most widely used methods use morphologic characteristics and determination of the metabolic properties of the unknown bacterium. These findings are compared with properties of known bacteria in a database and a presumptive identification made. Single enzyme tests have been commonly used to differentiate closely related species. For example, the enzyme catalase catalyzes the breakdown of peroxide into water and

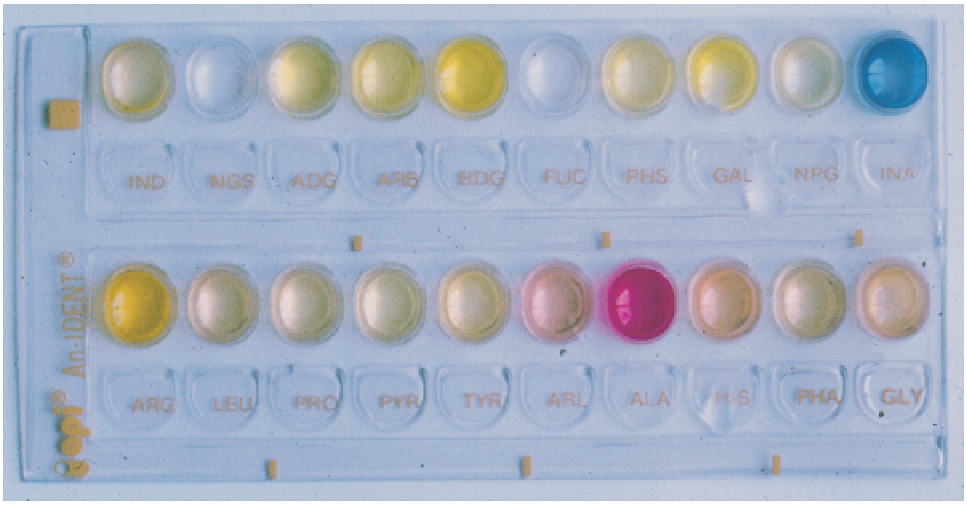

Fig. 3. An-IDENT anaerobe identification kit. Color changes indicate enzymes present capable of metabolizing different substrates. The metabolic profile can be used to make the identification.

oxygen. This test can be used to differentiate catalase-positive staphylococci from catalase-negative staphylococci, streptococci, and enterococci. An example for anaerobic bacteria is the use of *N*-α-benzoyl-DLarginine-2-naphthylamide (BANA) in conjunction with a colored complex to identify the presence of organisms with trypsin-like activity (*P. gingivalis*, *T. denticola*, *B. forsythus* (62, 63)). BANA can be used to differentiate asaccharolytic *P. gingivalis*, which has trypsin-like activity from *P. endodontalis*, which does not have trypsin-like activity.

A set of microtubes or microtiter plates with a number of substrates can be used to obtain a metabolic profile that can be used to make an identification of bacterial species (An-IDENT, API Analytic Products, Plainview, NJ, USA). An inoculum of a strain of bacteria in pure culture is placed into each well containing different substrates and incubated. The metabolic profile is compared with a data bank and the probability of identification is given (Fig. 3). Unfortunately, the results are often only presumptive and not definitive. For example, the metabolic profile might statistically suggest that the bacteria in question are 80% likely to be *F. nucleatum* and 20% likely to be *F. necrophorum*. Laboratories are increasingly using automated systems to identify pathogens. An inoculum is automatically transferred into the test wells and a photometer measures color changes related to metabolic (enzyme) activity of the bacterium with different substrates. The enzyme profile is analyzed in a computer database and a presumptive identification is made.

Immunological detection of microbes

Immunological methods take advantage of the specificity of antigen–antibody binding. These methods have been especially useful when the infecting bacteria are difficult to isolate, identify, or if a previous infection needs to be documented. These methods are relatively rapid with favorable specificity and sensitivity but do not permit further characterization. Monoclonal or polyclonal antibodies may be used for these tests. For example, the detection of a specific microbial antigen has been used in the 'Quelling reaction' to detect a bacterial capsule, the slide agglutination test to show clumping of *Salmonella*, and in gel counter electrophoresis to show an antigen in body fluids. Serum antibodies to specific microbes may be demonstrated using complement fixation (syphilis), direct agglutination (brucella), and direct hemagglutination (mononucleosis). Direct hemagglutination of sheep red blood cells has been used to differentiate *P. gingivalis* from other dark-pigmented bacteria that are negative for the reaction (64). Latex particles can be coated with either

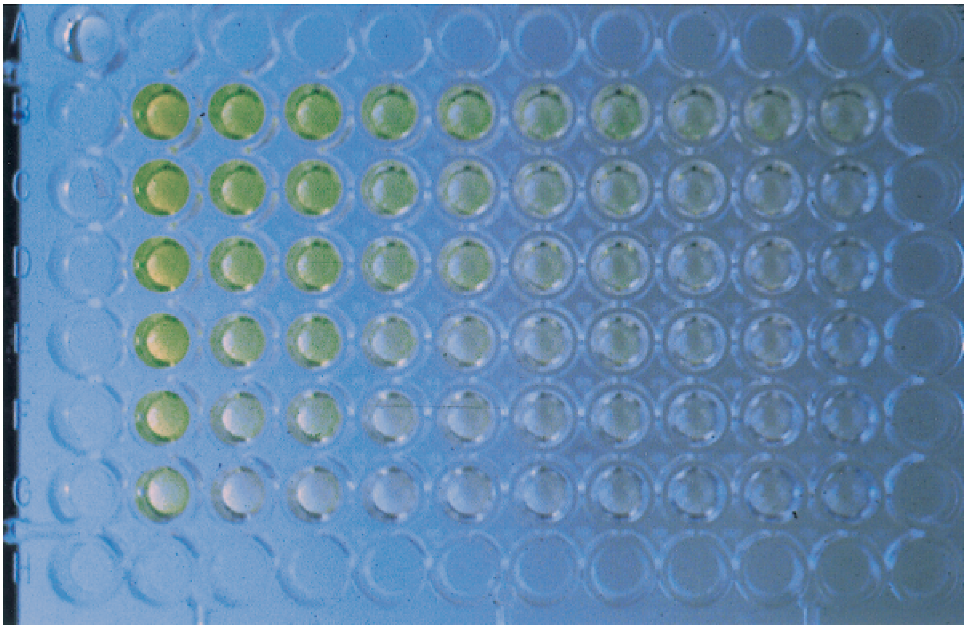

Fig. 4. Enzyme-linked immunosorbent assay (ELISA) showing results of dual titration of decreasing concentration of antibody in horizontal direction and decreasing concentration of antigen in the vertical direction.

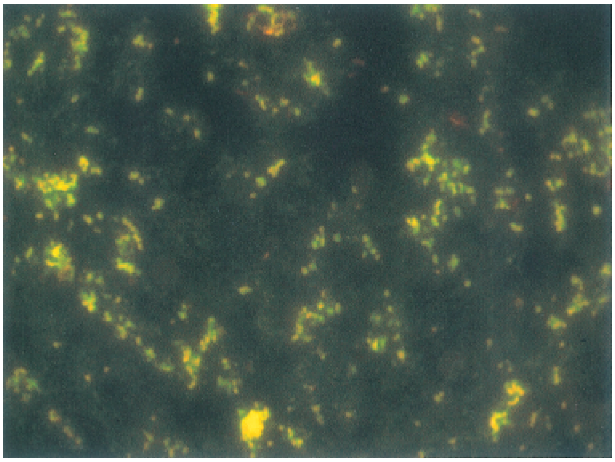

Fig. 5. Immunofluorescent microscopy of *Porphyromonas endodontalis*.

antigen or antibody and then used to test for the matching receptor. Latex agglutination of the particles has been used to test cerebral-spinal fluid for bacterial or fungal meningitis.

The enzyme-linked immunosorbent assay (ELISA) has often been used in endodontic research because of its sensitivity (35, 65–70). The ELISA is considered to be 1000 more sensitive than immunoassays using agglutination or precipitation of an antigen–antibody complex in a gel. For the ELISA, antibody specific for the antigen of interest is bound to the walls of a microtiter well. If the test sample has the complementary antigen, it will attach to the antibody. A second antibody that has a substrate that produces a colored product is then attached to the complex. A densitometer is used to detect and quantitate the amount of reactive antigen in microtiter plates (Fig. 4).

Direct immunofluorescence can be used with clinical samples to specific antigens. A clinical sample is incubated with a fluorescein-labeled antibody for a specific antigen on the microbe of interest. The labeled antibody absorbs ultraviolet light and emits visible fluorescence that can be seen using a fluorescence microscope (Fig. 5). This technique has been used in the field of endodontics to detect species of bacteria in endodontic samples (71–75). Immunofluorescent microscopy has the advantage over other immunological methods because the morphological structure of the cells can be viewed to reduce false-positive results caused by cross-reactivity of the antibody with a different organism. However, the ELISA is many times more sensitive in detecting small numbers of a specific organism.

The last decade has seen remarkable changes in clinical immunology in medicine. Many automated immunoassay machines are now available with even greater precision and sensitivity for the identification of microbes. These machines incorporate different immunoassays of detection including chemiluminescence, nephelometry, enzyme immunoassays, and electrophoretic separation. The spectrum of analytes includes traditional infectious disease, serology, drug testing, toxicology, oncology, and allergy. Many of the analytes (organisms) associated with endodontic disease are not in the test libraries.

Antibiotic susceptibility

Bacteria must be isolated in pure culture for antimicrobial susceptibility testing. The Etest (AB Biodisk, Culver City, CA, USA) for susceptibility testing of anaerobes has recently been used with promising results (76, 77). The Etest strips have logarithmic doses of the antibiotic on the side placed on the culture plate. The antimicrobial agents tested were penicillin V, amoxicillin, amoxicillin+clavulanic acid, clindamycin, metronidazole, and clarithromycin (76, 77). For the Etest, sterile cotton swabs are used to inoculate plates with the bacteria to be tested in pure culture. The Etest strips are then applied to the surface and the plates incubated in an anaerobic chamber for 48 h at 36–37°C

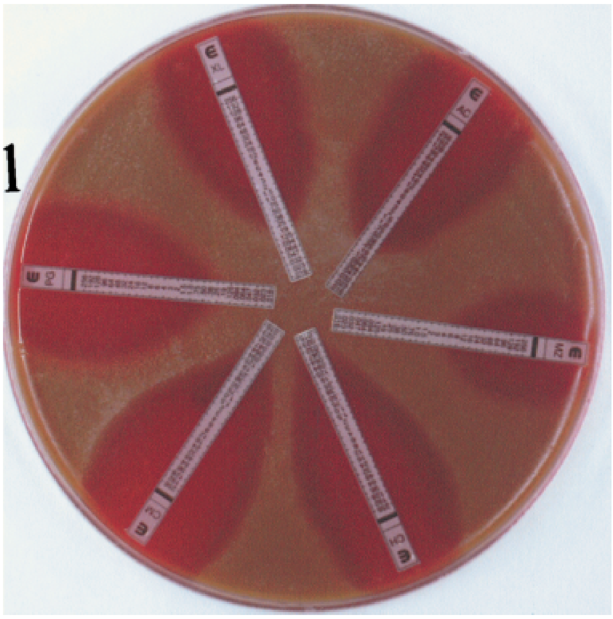

Fig. 6. Etest strips placed on agar showing zones of inhibition by each of six antibiotics.

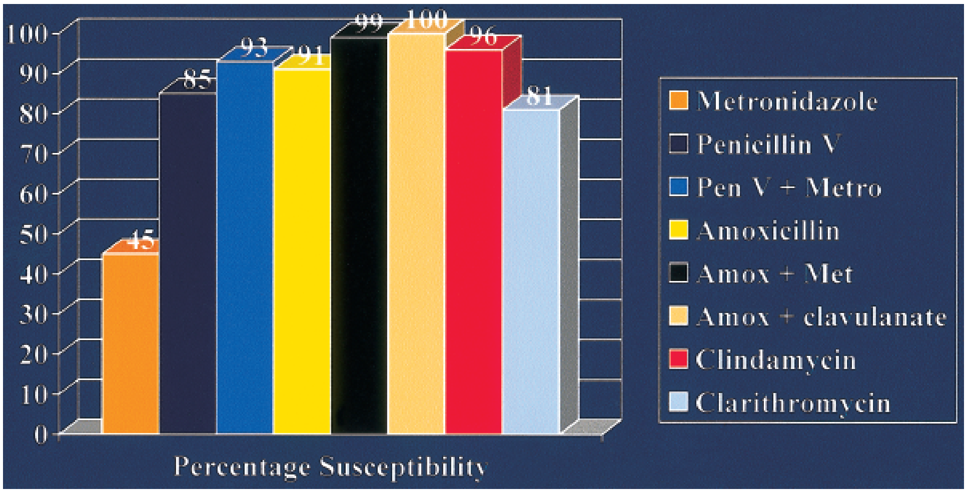

Fig. 7. Results of Etest antibiotic susceptibility.

for anaerobic bacteria and for 16–20 h at 36–37°C in an aerobic incubator for aerobic bacteria (Fig. 6). The susceptibility results are interpreted by comparing the minimum inhibitory concentrations (MIC) of isolates with MIC interpretive standards established by the National Committee for Clinical Laboratory Standard (NCCLS). Based on the results of these studies, it appears that penicillin V is still the antibiotic of choice for the treatment of endodontic infections in normal healthy patients (Fig. 7) (76, 77). Amoxicillin was slightly more effective but has a spectrum that may select for resistant organisms elsewhere in the body that are usually not associated with endodontic infections. Clindamycin and clarithromycin (an analogue of erythromycin) were shown to have good efficacy against the bacteria and are recommended when a patient is allergic to penicillin. For immunocompromised patients not allergic to penicillin, the combination of amoxicillin+clavulanic acid had the greatest efficacy (76, 77). Metronidazole is only effective against anaerobic bacteria and should only be used in combination with penicillin.

Detection of microbial DNA or RNA

Molecular microbiology has emerged as an area of interest in terms of both the number of tests performed and clinical relevance. Molecular techniques have reduced the dependency of the clinical microbiology laboratories on culture-based methods and created new opportunities for a clinical laboratory to affect patient care. Although culturing of organisms has been known as the ‘gold standard’ for the detection and identification of organisms, we now have the ‘DNA standard’ available for the detection and identification of microorganisms. In general, molecular methods are much easier, faster, and more precise than culturing methods. Duplication of nucleic acids *in vitro* may eventually replace biologic amplification (cultivation). One great advantage of molecular techniques is that viable organisms are not required. For molecular methods, the major goals for processing the clinical sample are to release nucleic acid from the organism, maintain the integrity of the nucleic acid, render the sample non-infectious, and remove inhibiting substances. A major disadvantage of molecular methods is that organisms are not cultivated so we do not know if the cells were viable and the cells are not available for other tests or research studies. For example, antibiotic susceptibility tests are not possible without viable bacteria. Molecular methods do not fulfill Koch’s postulates because the disease cannot be reproduced. Guidelines for establishment of causal relationships between microbes and disease have been suggested for molecular methods of detection and identification of microbes as follows (78).

Modification of Koch’s postulates for molecular methods

1. A nucleic acid sequence belonging to a putative pathogen should be present in most cases of an infectious disease.

2. Fewer, or no, copy numbers of pathogen-associated nucleic acid sequences should occur in hosts or tissues without disease.
3. With resolution of disease, the copy number of pathogen-associated nucleic acid sequences should decrease or become undetectable. With clinical relapse, the opposite should occur.
4. When sequence detection predates disease, or sequence copy number correlates with severity of disease or pathology, the sequence-disease association is more likely to be a causal relationship.
5. The nature of the microorganism inferred from the available sequence should be consistent with the known biological characteristics of that group of organisms.
6. Efforts should be made to demonstrate specific *in situ* hybridization of microbial sequence to areas of tissue pathology and to visible microorganisms or to areas where microorganisms are presumed to be located.
7. The sequence-based forms of evidence for microbial causation should be reproducible.

Unfortunately, the above guidelines for the molecular association of microbes with disease based on DNA/RNA sequence require a great deal of inference compared with Koch's postulates. Diseases such as Whipple's disease and hepatitis C have benefited from sequence analysis to detect the organism causing the disease. In Whipple's disease, an uncultivable bacillus seen in infected tissues with a microscope was detected and identified using polymerase chain reaction (PCR) and named *Tropheryma whippelii*. Hepatitis C (previously non-A, non-B hepatitis), an RNA virus, was detected using reverse transcriptase PCR (RT-PCR) and serological tests.

For molecular methods a sequence of base pairs (bp) must be known for the organism being surveyed and the possibility of cross-reaction of the probe with other organisms including unknown organisms is possible. Over 500 species of bacteria have been cultivated from the oral cavity. It is believed that many more species of bacteria are uncultivable using today's methods. Even if microbes cannot be cultivated, their DNA can be extracted from a sample and sequenced for comparison with known microbial sequences. Molecular methods avoid the many problems associated with taking a clinical sample and maintaining microbial viability until it is cultured. DNA can be extracted from dead or dieing cells to make identification of the species at the genetic level.

The basic strategy for diagnostic molecular microbiology is using microbial DNA or RNA to detect a relatively short sequence of nucleotide bases (target sequence) that is unique to the organism being detected or identified. This is accomplished by using a complementary sequence of nucleotide bases known as the probe or primer. For bacteria, the most commonly used sequences are located on the 16S ribosomal RNA gene. This gene has been especially useful because each bacterium has multiple copies and there are areas in the gene that are conserved and similar for all bacteria. Other sequences of bases in the 16S ribosomal RNA gene are variable among all bacteria but quite specific for each species. A probe or primer can be designed to complement the unique sequence of each species. The actual methods for detecting microbial DNA or RNA are variations of direct hybridization and the PCR.

DNA hybridization

Once the DNA from a microbe has been sequenced, a single-stranded molecular probe of complementary nucleotide bases can be designed that is unique for that target DNA of the microbe of interest. The probe is usually labeled with an enzyme, a fluorescent molecule, or a radioactive label that can be detected. The patient sample may be cultured to increase the number of microbes of interest and then treated to lyse the microorganisms and release the DNA. Single-stranded DNA is produced by alkaline denaturation of the double-stranded DNA. The denatured single-stranded DNA is immobilized on a nitrocellulose membrane for hybridization to the labeled probe. The membrane is washed to remove any unbound probe and the extent of hybridization is measured by the amount of probe remaining on the membrane. The direct use of nucleic acid probes requires a relative large amount of DNA compared with nucleic acid amplification methods such as the PCR. Hybridization has been used to study endodontic infections (79–83).

Polymerase chain reaction

PCR is an elegant tool for amplifying a nucleic acid sequence of DNA or RNA until there is enough nucleic acid to detect and identify the organism. PCR has the advantage over direct detection with molecular probes (hybridization) because PCR allows amplification of

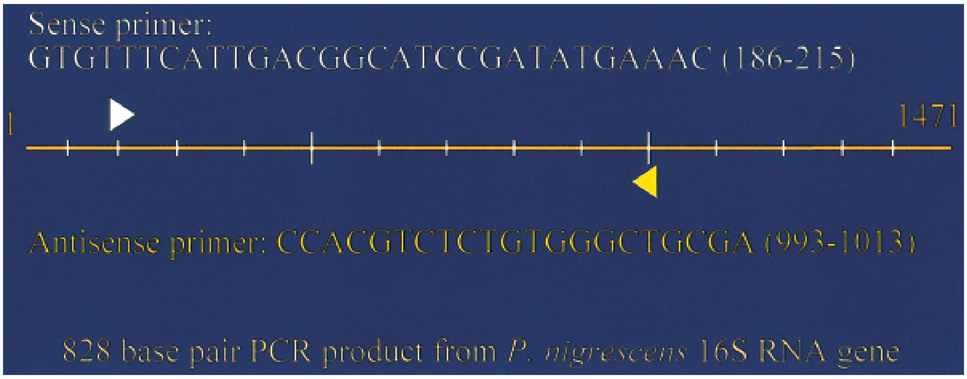
Sense primer:
GTGTTTCATTGACGGCATCCGATATGAAAC (186-215)
1
1471
Antisense primer: CCACGTCTCTGTGGGCTGCGA (993-1013)
828 base pair PCR product from *P. nigrescens* 16S RNA gene

Fig. 8. Amplification of *Prevotella nigrescens* (ATCC 33563) 16S Ribosomal RNA Gene (GenBank L16471).

the DNA or RNA target sequences (84). The target sequence is multiplied millions of times without having to culture the microbe. In fact the microbe does not even need to be viable as long as the DNA is intact. PCR is so sensitive it can detect the presence of less than 10 bacteria in a sample. Primer pairs, both sense and antisense, are used for bacterial PCR. They are designed from the variable sequences of the 16S rRNA gene of a specific species of interest. A primer is a small piece of synthetic DNA, called an oligonucleotide, that is known to complement the DNA sequence of the particular microbes gene being assayed (Fig. 8). The 16S rRNA gene sequence for most bacteria can be downloaded from GenBank, National Center for Biotechnology Information and aligned using Geneworks software (version 2.5, Intelligenetics, Campbell, CA, USA). PCR mixtures containing the extracted DNA from the clinical sample, the species-specific primers, polymerase, deoxynuclotide triphosphates (dNTPs), $MgCl_2$, and buffers are placed in a thermal cycler (85). The heat during each cycle causes the DNA to denature into single-stranded DNA that can anneal with the complementary dNTPs and form another amplicon with each cycle. With each 1-min thermal cycle, the number of DNA amplicons between the primers is doubled. With the amplicons doubling each minute, a million amplicons are produced in 20 cycles and billions of amplicons in 30 cycles. Instead of trying to find a genetic needle in a haystack, a haystack of needles (amplicons) is produced. By knowing the number of bp in a species-specific amplicon, it is possible to perform gel electrophoresis and by comparing the amplicon to a 100 bp ladder determine if the species of interest is present (Fig. 9). PCR can be used with liquid or membrane hybridization with labeled probes to demonstrate the presence of the specific amplicon. Because of its power in the detection and identification of microbes, PCR has become a popular tool for analyzing the bacteria in endodontic infections (83, 86–99).

Nested PCR was developed to increase both the sensitivity and the specificity of PCR. For nested PCR, there are two pairs of amplification primers and two rounds of PCR. The amplicon products of the first round are subjected to a second round of amplification with a second set of primers that anneal to an internal sequence of the first amplicon. Nested PCR has been used to detect treponemes in endodontic infections (93, 98, 100–102).

RT-PCR has been developed to amplify RNA targets. In this process complementary DNA (cDNA) is first produced from the RNA target by reverse transcription. The cDNA is then amplified by PCR. Initially problems arose with the technique because the RT enzyme was not heat stable. This was solved with the development of a thermostable DNA polymerase derived from *Thermus thermophilus* that functions properly as both an RT and a DNA polymerase. This technique has recently been used to detect the cytmegalovirus and Epstein–Barr virus in periapical lesions of teeth with intact crowns (103, 104).

Standard PCR is qualitative in that it can only detect or identify a gene sequence. Now quantitative PCR (Q-PCR) is available (105). Q-PCR will allow enumeration of bacteria, determination of viral titer, and measure gene expression (106). The technique uses two templates in each reaction. A DNA fragment (the competitor) of known concentration, and the clinical sample containing the target DNA are amplified. Because the amplification occurs simultaneously, both

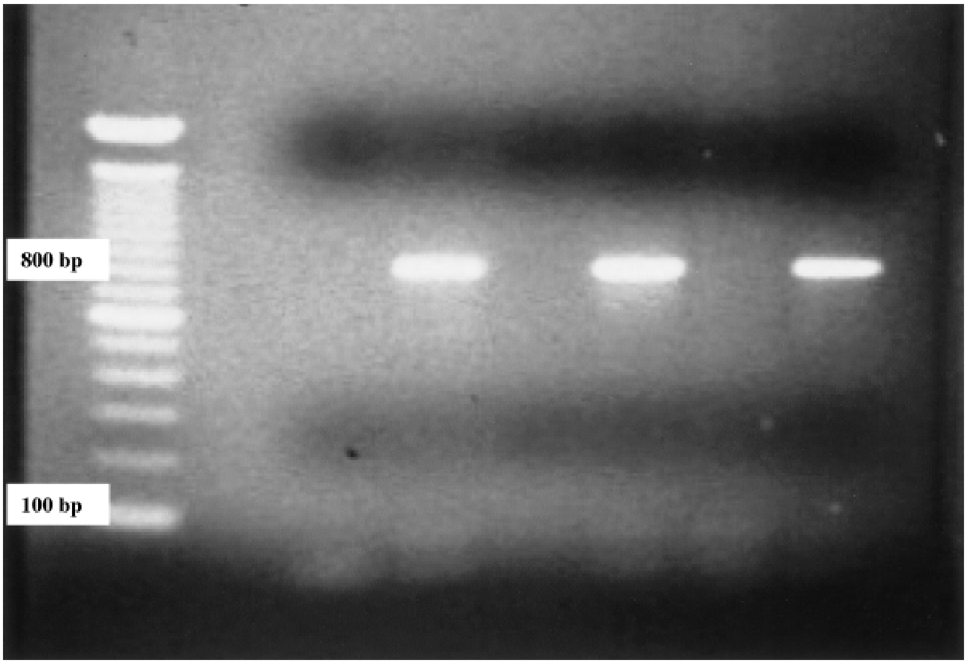

Fig. 9. Eight hundred and twenty eight base pair PCR product from *Prevotella nigrescens 16S RNA gene.*

fragments compete for the same set of primers. Early Q-PCR involved stopping the PCR and generating standard curves. More recently, fluorescent probes are used with the PCR reaction in microtiter plates with the relative amounts of fluorescence quantified in real time. As the number of amplicons increases, fluorescence increases proportionally. The ratio of the amounts can then be compared to determine the initial concentration of the target DNA.

Molecular arrays of DNA probes potentially have a great future. Microarrays are an ordered set of unique DNA probes molecules attached to a solid surface. Each array may have hundreds or thousands of probes typically less than 200 μm in diameter. About 10 000 DNA probes (PCR products) can be printed on a microscope slide. Microarrays can be used to detect DNA sequences and potentially used to detect the presence of unique DNA sequences for identification of specific species and even virulence factors.

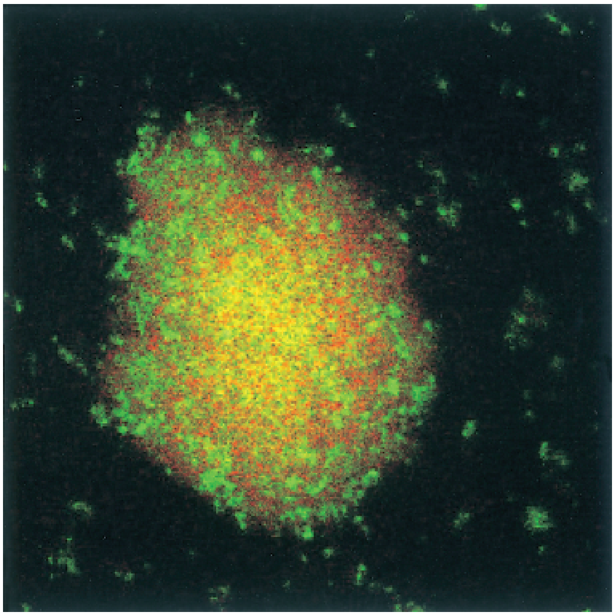

Fig. 10. Coaggregation of *S. hemolyticus/E. lentum* as seen with a confocal microscope (courtesy of S. Khemaleelakul).

Traditional vs. molecular methods

Identification of pathogens has generally resulted from a series of research accomplishments involving microscopy, culturing, immunological assays, and now molecular methods. Because of the spectacular nature of molecular techniques, some investigators have suggested that they will soon completely replace the need for microscopy, culturing, and immunological assays (107). However, a review of the literature continues to demonstrate the importance of each of these investigative modalities. For example, in medicine the diagnosis of leprosy caused by *Mycobacteriam leprae* and Lyme's Disease caused by *Borrelia burgdorferi* can be made microscopically. In the field of endodontics, light microscopy, dark-field microscopy, electron microscopy, and the confocal microscope continue to be important investigative tools (60, 108–112). Use of microscopes, especially the confocal microscope, to study coaggregation may provide significant insight into microbial pathogenicity in endodontic infections (Fig. 10).

The discovery that pseudomembranous colitis could be neutralized by clostridial antiserum led to the finding that *C. difficile* was the responsible agent (107). Immunological assays have provided indirect evidence of the microbes responsible for producing endodontic infections (113–116). Molecular methods have improved our detection of organisms that are in low numbers and those with fastidious growth requirements but may not be better than culturing for those organisms with defined growth conditions already in common use (80, 83, 91, 117, 118).

The combination of conventional and molecular techniques may be used to identify and determine the prevalence of an organism in endodontic infections. For example, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and PCR were used to differentiate *Prevotella intermedia* from *P. nigrescens* (87, 119). PCR identification demonstrated that *P. nigrescens* was more prevalent than *P. intermedia* in cultures from endodontic infections. In addition, five strains originally cultivated and identified as *P. intermedia* using conventional analysis could not be identified using PCR as either *Prevotella intermedia* or *P. nigrescens*. The 16S rRNA gene from the unknown bacteria was cloned into plasmid vector pCR II and then transformed into *Escherichia coli* (118). Restriction enzyme digestion and gel electrophoresis then gave an amplicon that was sequenced and identified as *P. tannerae*. Because of fastidious growth requirements, *P. tannerae* is rarely cultivated from clinical samples. When clinical samples were tested using PCR and primers specific for *P. tannerae*, 60% of the samples were positive for *P. tannerae* (118). SDS-PAGE (protein fingerprint) has been used to differentiate cultivated strains of *P. nigrescens*. SDS-PAGE of whole-cell protein of *P. nigrescens* isolated from either the coronal or apical segments of the same root canal have been shown to be two different strains based on the presence of unique proteins in each strain (120).

Nucleotide sequence analysis of the bacterial 16S rRNA gene has expanded our knowledge of the phylogenetic relationship of bacteria in endodontic infections. In addition to bacteria, phylogenetic analysis and precise identification has been used with treponemes, fungi, and viruses in endodontic research (85, 89, 90, 95, 97–99, 101, 103, 118, 121).

Although databases with microbial sequences are rapidly growing with new input of sequences, the current databases contain an insufficient number of entries to identify many species. There is no simple method for the integration of immunological, epidemiological, pathophysiological, and clinical data with the sequence data for taxon boundaries. Microbial isolation and culturing still are of great importance for studying microbial diseases. Growth of an organism in pure culture allows the use of microscopy, immunological studies, metabolic analysis, antibiotic susceptibility testing, and the use of experimental models. As powerful as the molecular techniques are in detecting the presence of specific microbes, microscopy, isolation/cultivation, immunological studies, and biochemical tests still are of great importance in future endodontic research.

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